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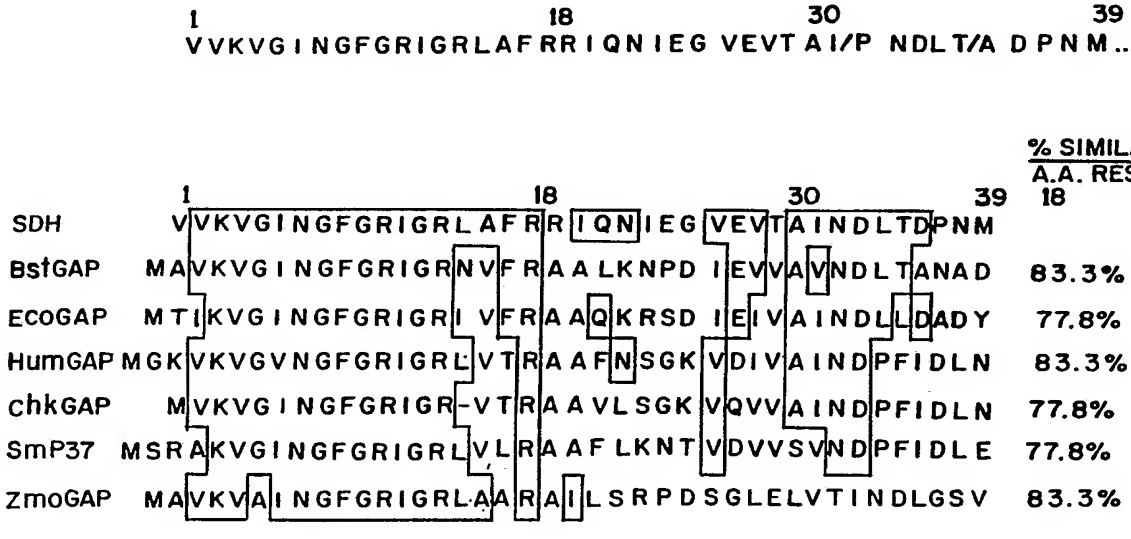

(57) Abstract

A novel streptococcal surface protein principally characterized by fibronectin and lysozyme binding activity, but also having glyceraldehyde-3-phosphate dehydrogenase activity, ADP-ribosylating activity and ADP-ribosyl transferase activity are described. It is particularly useful for the preparation of vaccines to protect against streptococcal infections.

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MULTIFUNCTIONAL SURFACE PROTEIN OF STREPTOCOCCI

This invention was made with government support under Grant Number AI-11822 awarded by the National Institutes of Health. The Government has certain rights in the invention.

RELATED APPLICATION

This application is a continuation in part of commonly owned and copending application serial number 07/913,732 filed July 15, 1992 which is, in turn, a continuation of application serial number 07/818,170 filed January 8, 1992. The latter application is now abandoned.

This invention relates to a surface protein of streptococci which is involved in early colonization of the pharyngeal mucosa. More specifically, it relates to a multifunctional protein which is on the surface of streptococci, including pathogenic streptococci, such as Streptococcus pyogenes and is particularly characterized by its ability to bind fibronectin, lysozyme, and the cyclosketal proteins myosin and actin as well as by its enzyme activity, specifically dose dependent dehydrogenase activity with glyceraldehyde-3-phosphate (GADPH). The molecule also functions as an ADP-ribosylating enzyme and as an ADP-ribosyl transferase.

It is concerned also with therapeutic compositions and uses of the surface protein including, for example vaccines prepared from the whole protein and segments thereof, particularly conserved segments having activity similar to that of the protein.

BACKGROUND OF THE INVENTION

Mammalian diseases, especially human diseases caused by streptococcal infection with bacteria such as Streptococcus pyogenes are a significant health problem. In the United States alone, 25 to 35 million cases of group A streptococcal infections, which primarily afflict school age children are reported annually (1). The high incidence and potential severity of streptococcal infections provide impetus for development of an effective and safe vaccine to prevent streptococcal related infections.

It has now been discovered that there is a streptococcal surface dehydrogenase (SDH) protein on the surface of streptococci from several serological groups such as group A type 6 streptococci which has both enzymatic activity and a binding capacity for a variety of proteins. In the earlier applications in this series, this surface protein was referred to as MF6, that being the laboratory code designation assigned to it when it was initially isolated, purified and characterized. It is now referred to as SDH since one of its principal characteristics is that it is Streptococcal Surface Dehydrogenase. More specifically, it is a member of the class of proteins which manifest glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity.

GAPDH proteins, as the name implies, are a class of dehydrogenase enzymes intimately involved in mammalian physiological reactions. Generally, members of the class are found in the cytoplasm, but some have been found associated with membranes and cellular cyclosketal structures of eukaryotes. The glycolytic enzyme of this

invention is believed to be unique in that it is a surface protein of prokaryotes. No other such GAPDH protein has previously been described.

The GAPDH protein of this invention has some structural characteristics similar to other proteins of the GAPDH family. For example, over 80% of the NH_2-terminal 18 of 39 amino acids are identical to the GAPDH family of enzymes. However, it differs in many other respects, as will be explained hereinafter. It is, therefore, a novel product which has not heretofore been isolated and characterized.

A detailed characterization of purified SDH has disclosed that its native conformation is probably a tetramer with a molecular weight of about 156 kDa. The molecular weight of the protein by mass spectrometric analysis is about 35.8 kDa. By SDS PAGE, it is about 39.2 kDa.

The protein has been identified on the surface of Groups A, B, C, E, G, H and L streptococci utilizing affinity purified anti-SDH antibodies. The protein exhibited a dose dependent dehydrogenase activity on glyceraldehyde-3-phosphate (G-3-P) in the presence of beta nicotinamide adenine dinucleotide (NAD). The multifunctional activity of SDH was revealed by its ability to bind fibronectin and lysozyme as well as the cytoskeletal proteins myosin and actin. The binding activity of SDH to myosin was found to be localized to the globular heavy meromyosin domain. SDH did not bind to streptococcal M protein, tropomyosin or the coiled-coil domain of myosin. The multiple binding capacity of SDH especially in connection with cycloskeletal proteins, in conjunction with its GAPDH activity indicates a role

in the colonization, internalization and the subsequent proliferation of streptococci. Trypsin treatment of whole streptococci resulted in a marked reduction in their reactivity to SDH antibodies. The inability to remove SDH from the streptococcal surface after washing in 2 M NaCl or 2% SDS indicates that the protein is not peripherally associated but tightly bound to the cell. These data all indicate that the protein is a surface GAPDH molecule on the streptococcal cell.

The novel SDH is obtained by solubilizing the selected streptococcus with lysin to produce a mixture containing SDH. The SDH may be isolated from the mixture by any of a number of convenient methods known to the skilled artisan including the method illustrated below.

It may also be produced by transforming an organism such as E. coli with an appropriate gene so that the E. coli will express SDH.

The following abbreviations are employed in the description of this invention:

NAD: beta nicotinamide adenine dinucleotide
PVDF: polyvinylidine difluoride
EDTA: Eyhylenediamine tetra acetic acid
PMSF: Paradimethyl sulfonyl fluoride
TLCK: N-p-tosyl-L-lysine chloro-methyl ketone
SDS: Sodium dodecyl sulfate
Mono Q FPLC: Mono Q(Trade Name) Fast protein liquid chromatography
Superose 12 FPLC: Superose-12(Trade Name) Fast protein liquid chromatography
TSK-Phenyl HPLC: TSK-Phenyl (Trade Name) high performance/pressure liquid

chromatography

NADH: beta-nicotinamide adeninedinucleotide, reduced

ELISA: Enzyme linked immunosorbent assay

ELIDA: Trade name of Physica Inc.

Sephadex G-25 PD-10: G-25 PD-10: trade name of Pharmacia-LKB Inc.

HEPES: (N-[2-hydroxyethyl]piperizine-N'-[2-ethansulfonic acid])

RGDS: Arginine-Glycine-Aspartic acid-Serine (Arg-Gly-Asp-Ser)

G-3-P: Glyceraldehyde-3-phosphate

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

THE FIGURES

There follows a description of the figures.

Fig. 1: SDS-polyacrylamide gel (10%) analysis of SDH protein from M6 streptococci. Lane a: Lysin extract of D471 streptococci. Lane b: Precipitate of 65% $(NH_4)_2SO_4$ saturation of the lysin extract. Lane c: Precipitate of 85% $(NH_4)_2SO_4$ saturation of the supernatant after 65% precipitation. Lane d: Pooled Mono Q fractions at 0.28 M gradient elution. Lane e: Partially purified SDH from the Superose 12 column. Lane f: Purified SDH from Phenyl TSK column. Arrow marks on lanes a and b at 50 kDa indicate the position of M protein. Prestained marker protein mixture with molecular mass as indicated on the left margin. 200 kDa:Myosin(H-chain), 97.4 kDa: Phosphorylase b, 68 kDa:bovine serum albumin, 43 kDa: ovalbumin, 29 kDa: carbonic anhydrase, 18.0 kDa: lactoglobulin, 14.3 kDa: egg-white lysozyme.

Fig. 2: (a) The NH_2-terminal sequence of SDH. (b) Comparison of the NH_2-terminal amino acid sequence of SHD with the amino acid sequences of the known GAPDH molecules obtained frm the translated Genbank database. BstGAP- Bacillus stearothermophilus GAPDH, EcoGAP- Escherichia coli GAPDH, HumGAP- human GAPDH, GAPDH, ChkGAP- Chicken GAPDH, SmP37- Schistosomia mansoni 37 kDa protein (GAPDH) (54), ZmbGAP- Zymomonas mobilis GAPDH. Numbers on the right side of the figure indicate the percentage similarity of SDH with other GAPDH molecules with residues 1-18 and 1-39. The gap(-) between the 14th and 15th residue of the chicken GAPDH sequence was introduced to maximize homology.

Fig. 3: Lineweaver-Burk's double reciprocal kinetic analysis of GAPDH activity of SHD. 25 ug of SDH was assayed as function of G-3-P in the presence of NAD (100uM) in triethanolamine-phosphate-EDTA-DTT buffer at pH 8.6. The Km for G-3-P was estimated to be 1.33 uM, Vmax: 0.487 X 10^{-3} M NADH min-^{1}, intercept Y axis (1/Vmax): 2.05 and slope (Km/Vmax): 2.73. The inset shows the analysis based on Michaelis-Menten. Km: 1.22 mM and Vmax:0.466 X 10^{-3} M NADH min^{-1}. (b) Lineweaver-Burk's double reciprocal kinetic analysis of GAPDH activity of the 39 kDa protein. 25 ug of SDH was assayed as a function of NAD in the presence of G-3-P (2 mM) in the buffer system as described in (a). The Km for NAD was estimated to be 156.7 uM, Vmax:0.459 X 10^{-3} M NADH min^{-1}, Intercept on Y axis(1/Vmax) 2.18, and slope (Km/Vmax) 341.74. Km for NAD by the method of Michaelis-Menten as shown in the inset was estimated to be 148.86 uM and Vmax: 0.445 X 10^{-3} M NADH min^{-1}.

Fig. 4: (A) Coomassie Blue stain of SDS-gel and (B) Western-blot analysis of SDH with affinity purified anti-SDH antibodies suggesting a multimeric structure for the SDH molecule. Lanes a and d: Crude lysin extract. Lanes b and e: Purified SDH. Lanes c and f: Unboiled purified SDH in sample buffer without SDS and saturated wit NAD. Arrow mark indicates the position of a molecule of the size consistant with a tetrameric form of SDH. MW markers are indicated on the left margin (Details on each marker-see Fig. 1).

Fig. 5: Dot blot immunoanalysis to locate SDH on the streptococcal surface. The assay determines the extent of reactivity of affinity purified anti-SDH antibodies to surface exposed protein before and after 2% SDS, 2M NaCl and trypsin treatments. Dot blots were treated with LumiPhos-530 substrate (41) and developed on X-ray film. Densitometric reading of the image obtained on the X-ray film was expressed as an optical density in terms of arbitrary units measured on an image analyzer using the Dumas program (Drexel University, Philadelphia, USA). An internal linear standard curve for the optical density 0.008 to 1.333 was obtained for final densitometric analysis of the dot blot. Each bar representes the mean of four to eight separate readings $\pm$ S.D.

Fig. 6: GAPDH activity of whole streptococci. (a) The GAPDH activity was observed at 340 mn of whole M6 streptococci by determining the conversion of NAD to NADH in the presence of G-3-P. Details of the buffer system is described in materials and methods. (b) Activity of trypsinized M6 streptococci. (c) Inhibition of enzyme

activity of affinity purified anti-SDH antibodies (1:30 of 0.5 mg/ml for 3 hr at room temperature). Each bar represent the mean of three separate readings ± S.D.

Fig. 7: (A) Western-blot analysis of lysin extract of various streptococcal M types with affinity purfied anti-SDH antibodies at a 1:2000 dilution of 0.5 mg/ml stock. Purified SDH and an M negative (M^-) streptococci are also incuded in the analysis. (B) Western-blot analysis of mutanolysin extract of various grouping strains of streptococci using anti-SDH antibodies as described in (A).

Fig. 8: Competition kinetic enzyme-linked immunosorbent assays (kELISA) with immobilized SDH. Commercially available purified GAPDH from B. stearothermophilus, human erythrocytes and rabbit skeletal muscle were used to compete for the binding of affinity purified anti-SDH antibodies (1:1000 dilution of 0.5 mg/ml stock). Each curve represents the mean of three separate experiments with less than 5% standard deviaiton (not shown). Inset shows the Western blot of the reactivity of affinity purified anti-SDH antibodies with (a) streptococcal SDH and GAPDHs of (b) bacterial (B. stearothermophilus), (c) rabbit skeletal muscle and (d) human erythrocytes.

Fig. 9: Binding of ^{125}I-SDH to cytoskeletal proteins. (A) Coomassie Blue stained SDS-PAGE gel (10%) containing 5 ug protein of various cytoskeletal proteins, lysozyme and M6 protein (43). Lane a: rabbit skeletal myosin. Lane b: heaving meromyosin. Lane c: light meromyosin. Lane d: actin. Lane e: M6 protein. Lane f: S-2 fragment of heavy meromyosin. Lane g: egg white lysozyme. (B) autoradiograph of proteins in a duplicate

gel after transfer onto nitrocellulose and incubation with the ^{125}I-SDH. The proteins in each lane are as described in Fig. 9(A). MW markers on the left margin (Detail of each marker - see Fig. 1).

Fig. 10: Binding activity of SDH to fibronectin. (A) Coomassie stain of an SDS gel containing 5 ug of SDH and BSA. (B) Western-blot analysis of a duplicate gel showing the binding of fibronectin followed by anti-fibronectin to the SDH molecule. (C) Autoradiograph of a similar Western blot showing the binding of ^{125}I-fibronectin to the SDH protein. Lanes a, c & e - SDH. Lanes b, d, & f - BSA.

Fig. 11: ADP-ribosylation of SDH. Purified SDH (lanes 1, 5 and 9), crude streptococcal cell wall extract (lanes 2, 6 and 10), cytoplasm (lanes 3, 7 and 11) and membrane (lanes 4, 8 and 12) fractions were incubated with [^{32}P]NAD in ADPR buffer. The proteins were then separated on a 12% SDS gel and stained with (A) Coomassie blue. (B) Western blot analysis of a duplicate gel reacted with affinity purified anti-SDH antibodies and (C) autoradiography of a similar Western blot. MW marker is indicated on the left side.

Fig. 12: ADP-ribosyl transferase activity of SDH. SDH and various bindig proteins (2740) were incubated together in the presence of [^{32}P]NAD. Protein mixtures were precipitated, washed and resolved on 12% SDS-PAGE and stained with Coomassie blue and a duplicate gel was dried and autoradiographed. SDH was incubated with actin (lanes, 1 and 6), chicken egg white Lysozyme (lanes, 2 and 7), S-1 fragment of myosin (lanes, 3 and 8), fibronectin (lanes, 4 and 9), and plasmin (lanes, 5 and 10). MW markers are indicated on the left side.

The following Materials and Methods section is provided for convenience and ease of understanding the invention.

MATERIALS AND METHODS

Materials:

Human fibronectin was obtained from Boehringer Mannheim. Goat anti-human fibronectin and affinity purified rabbit anti-goat IgG coupled to alkaline phosphatase were obtained from Sigma. Pre-stained molecular weight standards were purchased from Bethesda Research Laboratories. PVDF membrane "Immobilon-P" was from Millipore. $Na^{125}I$ was from New England Nuclear. All other chemicals and reagents unless otherwise indicated were purchased from Sigma.

Bacteria:

Bacteria. Group A β-hemolytic streptococcal strains of various M types and standard strains used for streptococcal grouping were from The Rockefeller University culture collection (New York, NY) and are listed as follows: M2(D626), M4(D896), M5(Manfrando), M6(D471), M24(CS24), M29(D23), M41(C101/103/4), M57(A995), M58(D774), M60(D398), M⁻(T28/51/4); group A, J17A4 (an M- strain); group A variant, A486var; group B, 090R; group C, C74; group D, D76; group E, K131; group F, F68C; group G, D166B; group H, F90A; group L, D167B; and group N, C559.

Lysin extraction and location of SDH protein:

A crude extract containing the major surface proteins was prepared using the procedure of lysin extraction to remove the streptococcal cell wall as described before (2). Essentially, bacteria washed in 50 mM sodium acetate buffer, pH 5.5 were suspended in the same buffer containing 30% raffinose and 5 MM EDTA. Lysin is added to the suspension (1:100 dil; 360 Units) and incubated for 90 min at 37°C with end-to-end slow rotation. The resulting protoplasts sedimented at 15,000 X g for 30 min in a Sorvall centrifuge. The supernatant was saved, dialyzed against 25 mM Tris/HC1, pH 8.5,5mM EDTA, concetrated on Amicon PM-10 membrane (Amicon Corp) and used for further purification.

After lysin extraction, the pelleted protoplasts were resuspended and lysed in hypotonic buffer (2 MM sodium acetate, pH 5.5, containing 2 mM PMSF, 1 MM TLCK, 10 MM $MgCl_2$ and 10 ug/ml DNAse) followed by three freeze/thaw cycles. The membranes were sedimented at 100,000 x g for 45 min at 4°C. The membrane pellet and cytoplasmic extract in the supernatant were analyzed with Coomassie blue stain after separation on SDS gel. Membranes were further treated with 1.5 M sodium chloride or 100 mM sodium carbonate, pH 11.3 to determine the nature of association of SDH protein with the membrane.

To determine whether this protein is surface exposed, lysin extraction of trypsinized bacteria was carried out as described earlier (3). Briefly, washed bacteria were suspended in 100 mM NH_4HCO_3 and digested with trypsin (250 ug/ml) at 37°C for 3 hr, after which the trypsin was inactivated by the addition of soybean trypsin inhibitor (200 ug/ml). Lysin extracts of

trypsinized and control non-trypsinized bacteria were compared for the loss or reduction in the size of SDH protein.

Purification of SDH:

Lysin extraction was used as the starting material for the purification of the SDH. The dialyzed, concentrated, lysin extract was precipitated at 60% saturation of ammonium sulfate at 4°C. The precipitates were centrifuged at 6,000 X g for 20 min and the supernatant was brought to 85% saturation of ammonium sulfate. The resulting precipitate was dialyzed against 25 mM Tris/HCl buffer pH 8.5, 5 mM EDTA and passed over to Mono Q FPLC column (Pharmacia LKB Biotechnology Inc.) equilibrated with the same dialyzing buffer. After the column was washed with 5 column volumes of starting buffer, bound proteins were eluted with a 50 ml linear NaCL gradient from 0 to 300 mM. Fractions containing SDH were pooled and dialyzed against the 35 mM Tris/HCl/EDTA buffer and rechromatographed on the Mono Q column. Fractions containing SDH were then pooled, and concentrated to a volume 1.0 ml on Centricon concentrator (cutoff mw 10,000 kDa). The concentrated sample was applied to a Superose 12 FPLC column (Pharmacia LKB Inc) pre-equilibrated with 50 mM Tris/HCl pH 8.5 containing 0.3 M NaCl and 5 mM EDTA. Fractions containing SDH protein were pooled, dialyzed against 0.025 M Tris/HCl buffer pH 8.5 containing 1.0 M $(NH_4)_2SO_4$ and applied to a TSK-phenyl HPLC column (Bio-Rad Laboratories, Richmond CA) preequilibrated in the same buffer. Theprotein was eluted by a decreasing linear gradient of $(NH_4)_2SO_4$ from 1.0 M to 0.0 M. The purity of the final product was determined by Coomasie blue stain of the purified protein on SDS gel and by the analytical

procedures. Purified material was stored at 4°C after dialyzing against 0.025 M Tris/HCl pH 8.5 for various protein binding experiments or at -70°C for longer storage.

Analytical Procedures (NH_2-terminal sequence and amino acid composition):

NH_2-terminal amino acid sequence was determined according to the method of Matsudaira et al (4). Briefly, the purified SDH was separated on a pre-electrophoresed 10% acrylamide-SDS gel under non-denaturing condition and then transferred to PVDF Immobilon-P filter pre-wetted in methanol. Protein was visualized by 0.05% Coomassie blue in 50:40 methanol, water, acetic acid solvent mixture. The blots were destained in methanol: water: acetic acid (50:40:10). The portion of the membrane containing the SDH band was excised and subjected to automated Edman degradation on an Applied Biosystem model A470 sequenator. Each band contained about 2-3 ug protein as determined by BCI protein estimation method (Pierce). For amino acid composition, the PVDF membrane containing the SDH was stained with 0.1% Ponceau-S (Sigma) in 1% acetic acid. The section of membrane containing the protein band was excised and destained with water. This section of membrane was hydrolyzed in 6N HCl/phenol at 110°C for 22 hr. Amino acids were separated on Waters Novapek C8 column analyzed with Waters Maxima software, 510 pump and 490 detector. Cysteine content was analyzed also from the PVDF bound carboxyamide methylated protein as described by Crestfield (5). All analyses were performed by Protein Biotechnology Facility of the Rockefeller University.

Molecular Mass Determination:

Molecular mass of the purified protein was determined in the department of Mass Spectrometry and Gas Phase ion chemistry of The Rockefeller University using the modified method of matrix-assisted laser desorption technique (6).

Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) Activity:

GAPDH assay was carried out according to the method originally described by Ferdinand (7) with a minor modifications. Since GAPDH catalyzes the oxidative phosphorylation of D-G-3-P to form 1,3-diphosphoglycerate in the present of NAD^+ and inorganic phosphate, the assay solution was made of triethanolamine (40 mM), Na_2HPO_4 (50 mM) and EDTA (5 mM). Disposable semi-micro 1.5 ml capacity spectrophotometer cuvette (VWR) contained 7ul G-3-P (Sigma, 49 mg/ml), 100 uM NAD (Boehringer Mannheim) and assay buffer to a final volume of 1.0 ml after the addition of enzyme source with pH of the mixture being 8.6. Different concentrations of SDH were used to plot the standard curve for the absorbance at 340 nm per minute as a measure of conversion of NAD to NADH using Spectronic 3000 spectrophotometer (Milton Roy).

Enzyme Kinetics:

Kinetics of enzymatic reaction of SDH were made with varying concentrations of NAD and a fixed concentration of G-3-P and vice versa to determine respectively the Km and Vmax for NAD and G-3-P. The results were recorded as rate analysis of NADH release at every half second for a period of 1 min at 340 nm. The molar extinction

coefficient of NADH 6.22 X 10^3 (8) was used to convert absorbance (340 nm)/min to NADH M/min. The kinetic coefficient were estimated from the secondary plots of intercept of primary Lineweaver-Burke plots with respect to each substrate. The specific activity of the enzyme (units/mg) was measured using the equation:

Sp activity = $v(1+m/S)_{NAD}(1+Km/S)_{G-3-P}$ where v = u moles NADH/min/mg of enzyme.

Specific activity of GAPDH activity for SDH was measured in the lysin extract, ammonium sulphate precipitate and pooled fraction at various purification stages.

Rabbit Immunization and affinity purification of immune sera:

New Zealand white rabbits were immunized subcutaneously with 200 ug of purified SDH emulsified in Freund's complete adjuvant (1:1) at multiple sites. Rabbits were boosted once with 200 ug of this protein in Freund's incomplete adjuvant (1:1). All rabbits were bled 3 weeks after the first and 10 days after the second immunization. All sera were filter sterilized and stored at 4°C.

To purify SDH specific antibodies from the polyclonal sera, 2.0 mg of purified SDH was linked covalently with free amino group of gluteraldehyde-activated affinity adsorbent as described before (9). Anti-SDH rabbit sera (2-3 ml) was adsorbed to and eluted from the SDH-beads column, dialyzed, concentrated and stored as described before (9). These antibodies were further purified on Protein A column (Pharmacia LKB)

essentially using the same buffer described above (9). The monospecificity of anti-SDH was first checked on Western blot as described above.

Dot-Blot Immunoassay to determine location of SDH:

The surface location of SDH was determined with the monospecific antibodies using a bacterial dot-blot immunoassay as previously described (10). Essentially, an overnight culture of strain D471 was adjusted to OD_{650} nm 1.0 with 50 mM Tris/HCl buffer, pH 8.5. Aliquots of this suspension were centrifuged and resuspended to the same volume of buffer containing either 2 M NaCl or 2% SDS, and rotated at room temperature for 1 h, centrifuged, and the respective supernatants were saved. After washing, the pellets were again adjusted to OD_{650} nm 1.0 with 50 mM Tris/HCl buffer, pH 8.5. In a separate experiment, the bacterial suspension in the Tris/HCl buffer was centrifuged, and the bacteria were suspended in 100 mM NH_4HCO_3 to $OD_{650\ nm}$ 1.0 and treated with trypsin (250 ug/ml) for 3 h at 37°C. Trypsin activity was inhibited with trypsin inhibitor as described above, and the bacteria were pelleted and resuspended in the Tris/HCl buffer to $OD_{650\ nm}$ 1.0 50 ul of each bacterial suspension was transferred to nitrocellulose paper using dot-blot assembly (Bio-Rad Laboratories, Richmond, CA). Reactivity of surface-exposed epitopes of the 39-kD protein was determined using affinity-purified anti-SDH protein antibodies (1:1,000 dilution of 0.5 mg/ml stock). For densitometric analysis of the dot blot, a duplicate blot was developed with Lumi-Phos™530 (Adamantyl-1,2-dioxetane phenylphosphate; Lumigen Inc., Detroit, MI), which undergoes enzyme (alkaline phosphatase)-catalyzed dephosphorylation to form a dioxetane anion that is converted ultimately into an excited state of the methyl

meta-oxybenzoate anion, the light emitter. The developer was then drained off, and the wet blot wrapped in Saran Wrap was exposed to x-ray film for 20 min and developed using conventional procedures. Densitometric analysis of each spot on the x-ray film was carried out on an image analyzer using the conventional procedures. Densitometric analysis of each spot on the x-ray film was caried out on an image analyzer using the Dumas program (Drexel University, Philadelphia, PA) interfaced with IBM computer.

GAPDH enzymatic activity of intact SDH protein on whole streptococci:

A whole cell assay was developed to determine whether SDH on the surface of streptococci serves as an active GAPDH enzyme. Different concentrations of trypsinized and non-trypsinized streptococci were incubated with and without G-3-P in presence of NAD in triethanolamine-phosphate-EDTA-DTT buffer as described above in a final volume of 1.0 ml for a period of 2 min at room temperature and centrifuged to pellet out the bacteria. The supernatants were analyzed for the conversion of NAD to NADH by recording absorbance at 340 nm. This enzymatic activity was also determined on streptococci preincubated with 1:50 dilution (1 mg/ml) of purified anti-SDH antibodies as prepared above to determine specific inhibition of enzymatic activity.

Polyacrylamide gel electrophoresis and Western blotting:

Electrophoresis, Western blotting of lysin extraction and protein samples at different purification stages were carried out as described earlier (2,3). Specific proteins bound to the nitrocellulose membrane

were probed and visualized with affinity purified anti-SDH antibodies (1:2000, 0.5 mg/ml) as described previously (2,3).

Presence of SDH on heterologous streptococcal M serotypes:

Lysin extracts of M serotypes 2, 4, 5, 6, 24, 29, 41, 57, 58, 60, and M^- were prepared as described (2). The muralytic enzyme mutanolysin (20 ug/ml; sigma Chemical Co.) was used to prepare cell wall extracts of each grouping strain suspended in 50 mM Tris/HCl buffer, pH 6.8, containing 5 mM EDTA, 5 mM $MgCl_2$, and 30% raffinose, and incubated at 37°C for 60 min under constant end-to-end rotation. Proteins in all the extracts were separated on SDS-PAGE and transferred to nitrocellulose. The blots were probed with affinity-purified anti-SDH protein antibodies as described above.

Relationship of GAPHDs from bacterial and mammalian origins with SDH:

The cross reactivity of GAPDHs isolated from rabbit skeletal muscle, human erythrocytes and B.stearothermophilus were determined both on Western blot and competitive ELISA as described below.

ELISA and Competitive inhibition:

Affinity purified antibodies were adjusted to a dilution that gave an ELISA reading of 1.0 at 405 nm after 60 min. ELISA was performed following standard procedures except that ELISA plates were coated with 100 ul/well of 1 ug/ml SDH for 3 hr at 37°C followed by overnight at 4°C.

Competition of GAPDH from different bacterial as well as mammalian origin containing cross reactive epitopes for the binding of Anti-SDH antibodies was performed as described previously (10). Briefly, ELISA plates were coated as described above with SDH. Optimum dilution of affinity purified antibodies as determined above was used. Competing GAPDH were serially diluted in antibody diluting buffer containing 0.05% Brij-35 pH 7.4 (10) at decreasing molar excess relative to SDH starting with 100 X molar excess. Anti-SDH antibodies were then added in each well and the plates were processed and finally developed and binding of these proteins was determined by kinetic ELISA as described (11) using ELIDA 5 microtitre plate reader Physics Inc. (20) at 405 nm.

Radioiodination of Proteins:

SDH was labeled with ^{125}I by the chloramine-T method using Iodobeads (Pierce Chemical Co.). The labeled protein was separated from free iodine by filtration over a column of Sephadex G-25 (PD-10, Pharmacia LKB Biotech Inc) and collected in 10 mM HEPES buffer saline pH 7.4 containing 10 mM $MgCl_2$, 2 mM $CaCl_2$, 50 mM KCl and 150 mM NaCl. The labeled protein was stored at -20°C in aliquots containing 0.02% NaN_3. Fibronectin and plasmin were labeled essentially by the same method. The specific activities of SDH, fibronectin and plasmin were, respectively, $2X10^5$, $1.0X10^6$ and $1.21X10^6$ CPM/mg.

Binding Studies:

The Binding activity of SDH and fibronectin was determined by the use of radioactive proteins. Egg white-lysozyme and/or cytoskeletal proteins (myosin, heavy meromyosin (HMM), light chain myosin (LMM),

tropomyosin, and actin) all of which obtained from Sigma, were electrophoresed on 10% SDS-PAGE gels and electroblotted on nitrocellulose paper. The blots were blocked in 10 mM HEPES buffer containing 15 mM NaCl, 0.5% Tween-20, 0.04% NaN_3 and 0.5% BSA pH 7.4 for 2-3 hr at room temperature and probed for 3-4 hr at room temperature in the same buffer containing ^{125}I-fibronectin, ^{125}I-plasmin at $3X10^5$CPM/ml. The probed blots were then washed 3-4 times with blocking buffer. Autoradiography were prepared by exposing the dried nitrocellulose blots to Kodak Blue Brand film with an intensifying screen for 36-48 hour at -70°C.

Lysin fractionation of Streptococci for ribosylation study:

An overnight culture of streptococci was washed and the cell wall was digested using the amidase enzyme lysin in 30% raffinose at pH 6.1 as described (2,3). After lysin extraction, which represents the cell wall fraction of the streptococci, the resulting protoplasts were further fractionated into cytoplasm and membrane after lyisng in a hypotonic buffer containing 10 mM $MgCl_2$ and DNAse (250 ug/ml) as described (3). Membranes were then separated from the cytoplasmic fraction by ultracentrifugation (100,00 X g, 45 minn, 4°C).

ADP-Ribosylation of SDH:

The ADP-ribosylation of SDH was performed as described (15) with slight modification. Briefly, the standard reaction mixture (0.2 ml) contained 100 mM Tris/HCl at pH 7.4, 10 mM dithiothreitol, 1 mM NADP, 10 mM thymidine (ADPR buffer). After the addition of 10 uM [alpha-^{32}P]NAD and 20 ug of purified SDH, the reaction

mixture was incubated for 1 hour at 37°C. The reaction was then stopped by the addition of 50 ul of 100% (w/v) chilled trichloroacetic acid (TCA), and allowed to stand for 30 minutes on ice after which time the precipitated proteins were separated by centrifugation (16,000 X g, 5 minutes at 4°C). The protein pellet was washed in absolute alcohol containing 1% of 5 M sodium acetate and dried in a Speedvac (Savant) to remove remaining TCA. The dried precipitates were dissolved in 50 ul of sample buffer and then subjected to SDS-PAGE (12% polyacrylamide) as described (2,3). The gel was dried, and autoradiograms were made with Kodax X-omat film using an intensifying screen at -80°C.

GAPDH Activity of ADP-ribosylated SDH:

The GAPDH activity of purified SDH and the ADP-ribosylated SDH was measured by the method originally described by Ferdinand (7) and modified as described (16). Briefly, the reaction was performed in a final volume of 1 ml containing 800-850 ul of buffer (40 mM triethanolamine, 50 mM Na_2HPO_4, 5 mM EDTA, pH 8.6), 100 uM NAD and the enzyme source (ADP-ribosylated and non-ADP-ribosylated SDH, 5 ug) dispensed in a 1.5 ml capacity microcuvette. The reaction was initiated with the addition of 7 ul glyceraldehyde (49 mg/ml). Absorbance at A_{340} nm showing the conversion of NAD to NADH was recorded over a period of 2 min.

Effect of Sodium Nitroprusside on the ADP-Ribosylation of SDH:

Sodium nitroprusside was freshly diluted in ADPR buffer (200 ul) to a final concentration of 2 mM and preincubated for 2 minutes at room temperature before the

addition of 30 ug of SDH and [^{32}P]-NAD to start the ADP-ribosylation reaction. At different time intervals, 40 ul aliquots were removed and precipitated with TCA. A parallel control representing the same quantity of SDH and [^{32}P]-NAD were incubated in the absence of sodium nitriprusside and aliquots were taken at the same time intervals as the test samples. Precipitated proteins were separated on SDS gel and autoradiographed. In a similar set of experiments, ADP-ribosylation was also performed using 200 ul of a streptococcal lysin extract in ADPR buffer incubated in the presence and absence of 2 mM sodium nitroprusside.

The results of the foregoing procedures are summarized below.

Purification of SDH protein receptor:

SDH protein, was precipitated from the lysin extract by first precipitating non-specific proteins at 60% saturation of ammonium sulfate followed by 85% saturation. The SDH was found in the 85% ammonium sulfate precipitate (Fig. 1). The dialyzed precipitate was applied to a Mono Q FPLC column and the proteins eluted with an NaCl gradient from 0 mM to 300 mM. SDH eluted at a salt concentration of about 280 mM. Fractions with fibronectin binding activity were pooled, dialyzed and further purified on a Superose-12 FPLC molecular sieving column. The small amount of contaminating proteins was removed by hydrophobic chromatography using a TSK-phenyl column. SDS-PAGE of the final preparation revealed a homogeneous protein with a molecular weight of 39 kDa. The total yield of purified protein from four liters of culture representing 6-8 gms wet weight of bacteria was 800 ug.

N-terminal Sequence and amino acid composition analyses:

NH_2-terminal amino acid sequence analysis of the purified SDH confirmed the homogeneity of the preparation resulting in a single amino acid at nearly all positions (Fig. 2a). Except for positions 31 and 35, a single amino acid was identified in the first 35 residues with the remaining four tentatively identified.

The amino acid composition of the purified protein indicated a high content of Asp/Asn (12.1%), followed by Ala (10.7%), Gly (10.3%), Val (10.2%), and Glu/Gln (8.4%). The mass of the purified protein (35,882 daltons) as determined by laser desorption mass-spectrometry was used to more precisely assign the number of residues/mol (Table 1).

Amino Acid Sequence and Composition Comparison:

When the sequence of the first 39 amino acids of SDH was compared to known sequences in the translated Gen-Bank database (Fig. 2b), significant identity was found with bacterial and eukaryotic GAPDHs. The identity within the first 18 residues was 77-83% with bacterial, eukaryotic, or fungal GAPDHs. This strong homology decreased over the remaining 21 residues with an overall identity of from 41-56% (Fig. 2b).

When the amino acid compositions of the various GAPDHs were compared, the methionine content of SDH was found to be significantly low (1.8 residue/mol) with relation to the eukaryotic (8.4 residue/mol) or other bacterial GAPDHs (7 residues/mol) (Table 1). Although the amino acid compositions of the remaining residues of SDH were found to be relatively close to that of the

other GAPDHs, sufficient differences were found that suggest that, except for the NH_2-terminal sequence, SDH is different from other reported GAPDHs.

GAPDH activity of SDH protein:

In the presence of G-3-P in triethanolamine buffer at pH 8.6, SDH showed a dose dependent conversion of NAD to NADH as observed by absorbance of the latter at 340 nm. Using 30 ug of purified SDH, variation of enzyme reaction rates with varying concentrations of G-3-P and NAD was determined. The results were analyzed both as Michaelis-Menten plots as well as double reciprocal plots according to Lineweaver-Burk (13) as shown in Figs. 3a and b. From these plots the Km for G-3-P and NAD was estimated to be 1.33 mM Vmax 0.487 X 10^{-3} M NADH min^{-1}. Fig. 5 shows the analysis based on the method of Michaelis-Menten. Km, 1.22 mM; and Vmax, 0.466 x 10^{-3} M NADH/min. (b) 25 ug of the 39-kD protein was assayed as a function of NAD in the presence of G-3-P (2mM) in the buffer system described above. The Km for NAD was estimated to be 156.7 uM; Vmax, 0.459 x 10^{-3} M NADH/min; intercept on y-axis(1/Vmax), 2.18; and slope (Km/Vmax), 341.74 Km for NAD by the method of Michaelis-Menten as shown in the inset was estimated to be 148.86 uM; and Vmax, 0.445 x 10^{-3} M NADH/min.

Determination of location of SDH on cell:

Antibodies to SDH were affinity purified on SDH-bound to activated gluteraldehyde beads followed by a protein A column. The resultant purified anti-SDH IgG recognized only the SDH protein band (Fig. 4). Dot-Blot immunoassay was applied to determine the location of SDH on streptococcal surface. Results revealed that trypsin

treated streptococci were markedly reduced in their reactivity to anti-SDH IgG (Fig. 5). To determine if the SDH protein is peripherally bound to the cell wall or tightly bound, the streptococcal cells were washed with 2M NaCl and 2% SDS. The results revealed that the SDH was not extractable by the high salt or ionic detergent.

Surface Enzyme activity of streptococci:

To determine if the GAPDH enzymatic activity found with the purified SDH protein was also present on the streptococcal surface, enzymatic studies were carried out using whole streptococci. The same concentration of substrates (G-3-P and NAD) used with the purified SDH were used with whole streptococci.

Data presented in Fig. 6 revealed a dose dependent GAPDH activity catalyzed by the whole organisms. As found with the purified SDH, the intact bacteria also did not catalyze the reaction in absence of the specific substrates G-3-P and NAD (Fig. 6a). The enzymatic activity on the whole organisms was also found to be partially (30%) but specifically inhibitable by anti-SDH IgG (Fig. 6c). Enzymatic activity was found to be decreased by 80% when trypsinized bacteria were used in the reaction mixture (Fig. 6b). The background 20% activity suggested an incomplete digestion of SDH protein by trypsin.

Prevalence of SDH protein in other M serotypes:

The ubiquitous nature of the SDH protein in different streptococcal M serotypes was determined by Western blot analysis of lysin extracts using affinity purified anti-SDH IgG. As shown in Fig. 7, SDH protein

was found in several serotypes. Furthermore, all were found to be of same molecular weight without any indication of size variation.

Relationship of SDH with GAPDHs of bacterial, animal and human origin:

The relationship of SDH with known GAPDHs was determined by both Western blot and competitive kinetic (k) ELISA using affinity-purified anti-SDH antibodies. Western blot (Fig. 8, insert) analysis with with SDH-specific antibodies revealed that GAPDH from bacillus, human RBCs, and rabbit muscle reacted weakly or not at all. This finding was further confirmed by competitoin ELISA (Fig. 8) showing that only a maximum of 20-25% inhibition of binding of anti-SDH antibodies to SHD could be achieved with 100 molar excess of these proteins, with the rabbit muscle GAPDH exhibiting the least activity. The fact that almost 20% inhibition is observed with 20 times molar excess of bacillus and human GAPDH may reflect the sequence homology observed at the NH_2 termini of these molecules (Fig. 2).

Binding property of SDH with lysozyme and cytoskeletal proteins:

Since many glycolytic enzymes have been shown to bind cytoskeletal proteins a determination was made as to whether SDH has a similar property. ^{125}I-SDH was used to probe a Western blot containing several cytoskeletal proteins. The results revealed that SDH binds to myosin and its globular domain (heavy meromyosin) and actin but not to the α-helical domain of myosin (light meromyosin) or tropomyosin. (Figs. 9a and b)

Binding of fibronectin to the SDH protein:

The fibronectin binding activity of SDH was determined both by using ^{125}I-labeled fibronectin or fibronectin-anti-fibronectin on a Western blot. The results revealed that the SDH protein was able to bind fibronectin in both assays (Figs. 10b and c).

Based on the results of all of the above, it has been determined that SDH is a major surface protein of streptococci, including group A streptococci and has both enzyme activity and multiple binding activity. No such protein has previously been detected, isolated and characterized. The novel surface protein is principally characterized by its ability to bind fibronectin, lysozyme and cycloskeletal protein as well as by its enzymatic activity as a GAPDH. Its molecular weight is approximately 39 kDa. The first fifteen amino acid residues at the amino terminal are:

Val-Val-Lys-Val-Gly-Ile-Asn-Gly-Arg-Ile-Gly-Arg-Leu-Ala-Phe

These first fifteen amino acid residues manifest 100% homology with the bacterial form of GAPDH and 80-90% homology with eukaryotic or fungal GAPDH. SDH is, however, significantly different from previously recorpted GAPDHs, because the high homology of the first fifteen amino acid residues is not preserved towards the carboxy end of the molecule and the amino acid composition varies appreciably from other GAPDHs.

SDH also functions as an ADP-ribosylating enzyme which, in the presence of NAD, is auto-ADP-ribosylated. It has been found that in a crude lysin extract of group

A streptococci containing a mixture of cell wall associated molecules, SDH is the only molecule that is ADP-ribosylated. Treatment of ADP-ribosylated SDH with the cytoplasmic fraction of Group A removed the ADP-ribosye of SDH which indicates the presence of SDH specific ADP-ribosyl hydrolase in the cytoplasmic compartment. Treatment of purified SDH or the crude lysin extract with sodium nitroprusside, which spontaneously generates nitric oxide, was found to stimulate the ADP-ribosylation of SDH in a time dependent manner. Both ADP-ribosylation and nitric oxide treatment inhibited the glyceraldehyde-3-phosphate dehydrogenase activity of SDH. In addition to its auto ADP-ribosylation activity, either purified SDH or whole streptococci with surface SDH were able to ADP-ribosylate specifically both chicken and human lysozyme, strong SDH binding proteins. These data show that SDH has both autoribosylation and ADP-ribosyl transferase activities.

SDH, as will be recognized by those skilled in the art, does not represent a single protien, but rather a class of surface proteins of streptococci, all of which have similar properties. The protein is involved in the colonization and probably in the internalization and proliferation of group A streptococci. One of the initial steps in the colonization of mucosal tissue by streptococci and subsequent infection by this bacteria, is the binding of the bacteria to fibronectin. Lysozyme is also believed to be involved in this binding step.

The enzyme activity of SDH may be involved in the binding of the bacteria to endothelial cells by reaction of an aldehyde reduction product of teichoic acid which

is a polyglycerol phosphate. The aldehyde function could bind the bacteria to the tissue surface by reaction with amino group on that surface.

Inhibition of this initial binding is, therefore, a major fuction in inhibiting or preventing streptococcal infection. Accordingly, antibodies to SDH which successfully compete with fibronectin and lysozyme for binding sites on the bacteria will inhibit the colonization of the pharyngeal mucosa by group A streptococci. Therefore the SDH and amino acid segments of the protein containing the appropriate antigenic determinants, for example those containing from about 6 to about 20 amino acid residues, are useful to inhibit streptococcal infection of mammals, including humans, by administering an amount of the selected product which will be effective to inhibit fibronectin binding and thereby inhibit colonization of the pharyngeal mucosa.

The proteins, polypeptides and peptides of this invention may be obtained by any of a number of known processes.

The protein can be isolated as described above. Alternativley, the protein or segments thereof can be prepared by recombinant DNA techniques. For example, the gene for the protein or an oligonucleotide for the desired segment can be inserted into a plasmid and the plasmid used to transform E. coli so that the bacteria will express the desired product.

Polypeptide and peptides within the scope of the invention containing, for example from about 6 to 20 or more amino acid segments, may be synthesized by standard solid phase procedures with appropriate amino acids using

the protection, deprotection and cleavage techniques and reagents appropriate to each specific amino acid or peptide. A combination of manual and automated (e.g., Applied Biosystem 430A) solid phase techniques can be used to synthesize the novel peptides of this invention. Altough less convenient, classical methods of peptide synthesis can also be employed. For background on solid phase techniques, reference is made to Andreu, D., Merrifield, R.B., Steiner, H. and Boman, H.G., (1983) Proc. Natl. Acad. Sci USA 80, 6475-6479; Andreu, D., Merrifield, R.B., Steiner, H. and Boman, H.G., (1985) Biochemistry 24, 1683-1688; Fink, J., Boman, A., Boman, H.G., and Merrifield, R.B., (June 1989) Int. J. Peptide Protein Res. 33, 412-421; Fink, J., Merrifield, R.B., Boman, A. and Boman, H.G., (1989) J. Biol. Chem. 264-6260-6267; each of which is hereby incorporated herein by reference.

The products of the invention are amphoteric. They can exist and be utilized as free bases or as pharmaceutically acceptable metallic or acid addition salts. Suitable metallic salts include alkali and alkaline earth metal salts, preferably sodium or potassium salts. Acid addition salts may be prepared from a wise variety of organic and inorganic acids including mineral acids, for example citric, lactic, maleic, tartaric, phosphoric and hydrochloric acids. These salts can be prepared by procedures well known to those skilled in the art.

For use as a vaccine, it is presently preferred to administer the selected product conjugated to a carrier such as cholera toxin B. Methods for preparing such

conjugates are known. One procedure is described by Bessen and Fischetti (14). Other carriers can be employed or the products can be used without a carrier.

The protein or segment thereof may also be administered as a hybrid protein expressed on a streptococcal surface utilizing the procedure of Pozzi et al (17,18).

Mice or other mammals including humans when immunized parenterally or orally have significant resistance to subsequent streptococcal challenges.

The presently preferred method for the administration of the vaccines of the invention is by the intranasal route, but the invention is not so limited. Other parenteral or oral prcoedures may be employed.

Typically, the patient to be protected will be treated with an amount of SDH or other product of the invention which is effective to elicit a protective immune response. The selected agent may be administered alone or in a pharmaceutically acceptable liquid or solid carrier in which it may be dispersed, dissolved or suspended. If, for example, the patient is to be treated intravenously, the peptide may be suspended as a free base or dissolved as a metallic salt in isotonic aqueous buffer. Other methods of treatment and pharmaceutically acceptable carriers will be apparent to the skilled artisan.

The proteins, polypeptides and peptides of this invention and the genes or oligonucleotides which are employed in their expression are useful as probes for genes and proteins. They are also useful to raise

antibodies by which specific strains of streptococci can be identified. For example in tests for mammalian infections.

5

TABLE 1

Comparison of Amino Acid Composition of SDH Protein from M Type 6 Streptococci with That of GAPDH from Different Species

	No. of residues/mol			
Amino Acid	SDH* (35.8)+	BSt (36.0)	Thaq (36.0)	RSM (34.0)
Asn/Asp	43.3	41	36	35.5
Glu/Gln	29.9	26	24	18.7
Ser	16.8	17	13	19.7
Gly	36.6	24	25	31.7
His	7.2	9	10	9.8
Arg	15.5	15	15	10.1
Thr	27.0	18	22	21.6
Ala	38.1	38	41	32.6
Pro	13.6	11	12	11.9
Tyr	9.1	8	10	8.6
Val	36.5	43	29	31.2
Met	1.8	7	7	8.4
Ile	22.4	19	22	15.3
Leu	23.4	26	30	17.8
Phe	13.8	5	7	12.9
Lys	21.4	23	23	23.9
Cys§	3.1§	2	1	3.0
Trp	ND	2	3	ND

Bst, B.stearothermophilus (19); Thaq, Thermus aquaticus (52); RSM, Rabbit skeletal muscle (20).

* Mean of three determinations.

+ Molecular mass of SDH protein (35,882) was measured by

laser desorption mass spectrometry.

§ Determined by carboxy amidomethylation method (5).

The publications identified in this specification are all incorporated herein by reference.

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WHAT IS CLAIMED IS:

1. Streptococcal surface dehydrogenase and segments thereof capable of binding streptococci to fibronectin, lysozyme and cycloskeletal proteins, having glyceraldehyde-3-phosphate dehydrogenase activity, ADP-ribosylating activity and ADP-ribosyl transferase activity.

2. A vaccine effective to inhibit colonization of mucosal tissue by streptococci containing, together with a pharmaceutically acceptable carrier, a streptococcal surface hydrogenase or a segment thereof in an amount which is effective to inhibit said colonization, said hydrogenase being capable of binding streptococci to fibronectin, lysozyme and cycloskeletal proteins, having glyceraldehyde-3-phosphate dehydrogenase activity, ADP-ribosylating activity and ADP-ribosyl transferase activity.

3. A method of treating a mammal to inhibit colonization of mucosal tissue by streptococci in a mammal in need of such inhibition which comprises administration of a streptococcal surface hydrogenase or a segment thereof capable of binding streptococci to fibronectin, lysozyme and cycloskeletal proteins, having glyceraldehyde-3-phosphate dehydrogenase activity, ADP-ribosylating activity and ADP-ribosyl transferase activity in an amount sufficient to effect such inhibition.

4. A method of obtaining a streptococcal surface dehydrogenase which comprises solubilizing streptococci with lysin to produce a mixture containing the dehydrogenase and isolating the dehydrogenase from the

mixture said dehydrogenase being capable of binding streptococci to fibronectin, lysozyme and cycloskeletal proteins, having glyceraldehyde-3-phosphate dehydrogenase activity, ADP-ribosylating activity and ADP-ribosyl transferase activity.

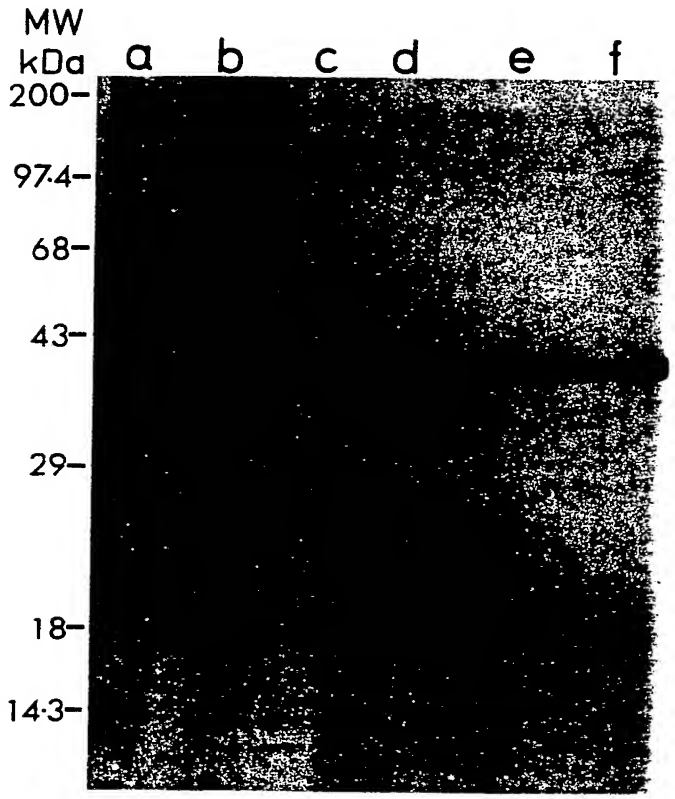

FIG. 1

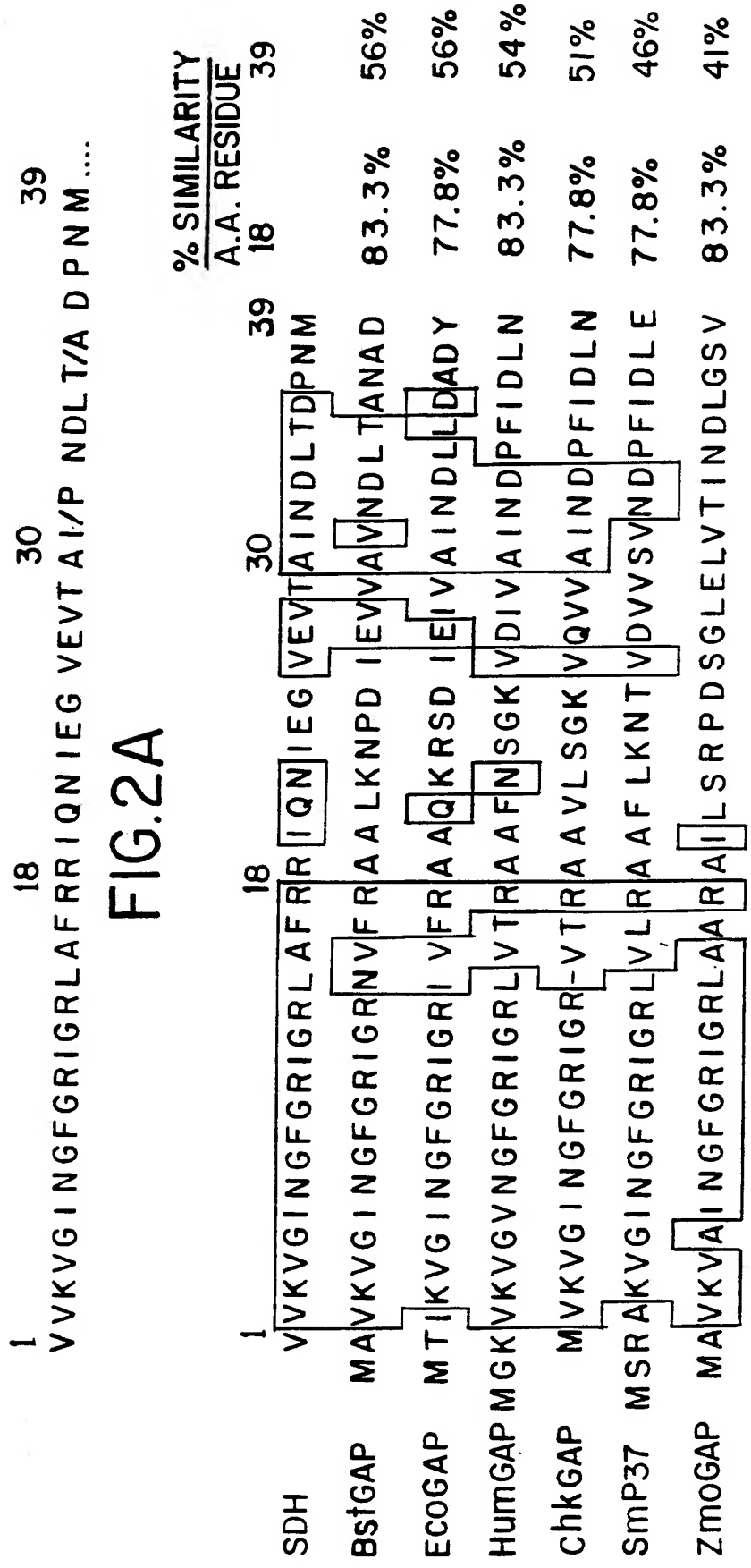
1 18 30 39
V V K V G I N G F G R I G R L A F R R I Q N I E G V E V T A I / P N D L T / A D P N M ....
% SIMILARITY
A.A. RESIDUE
18 39
SDH
BstGAP
ECOGAP
HumGAP
ChkGAP
SmP37
ZmoGAP
83.3% 56%
77.8% 56%
83.3% 54%
77.8% 51%
77.8% 46%
83.3% 41%

FIG.2A

FIG.2B

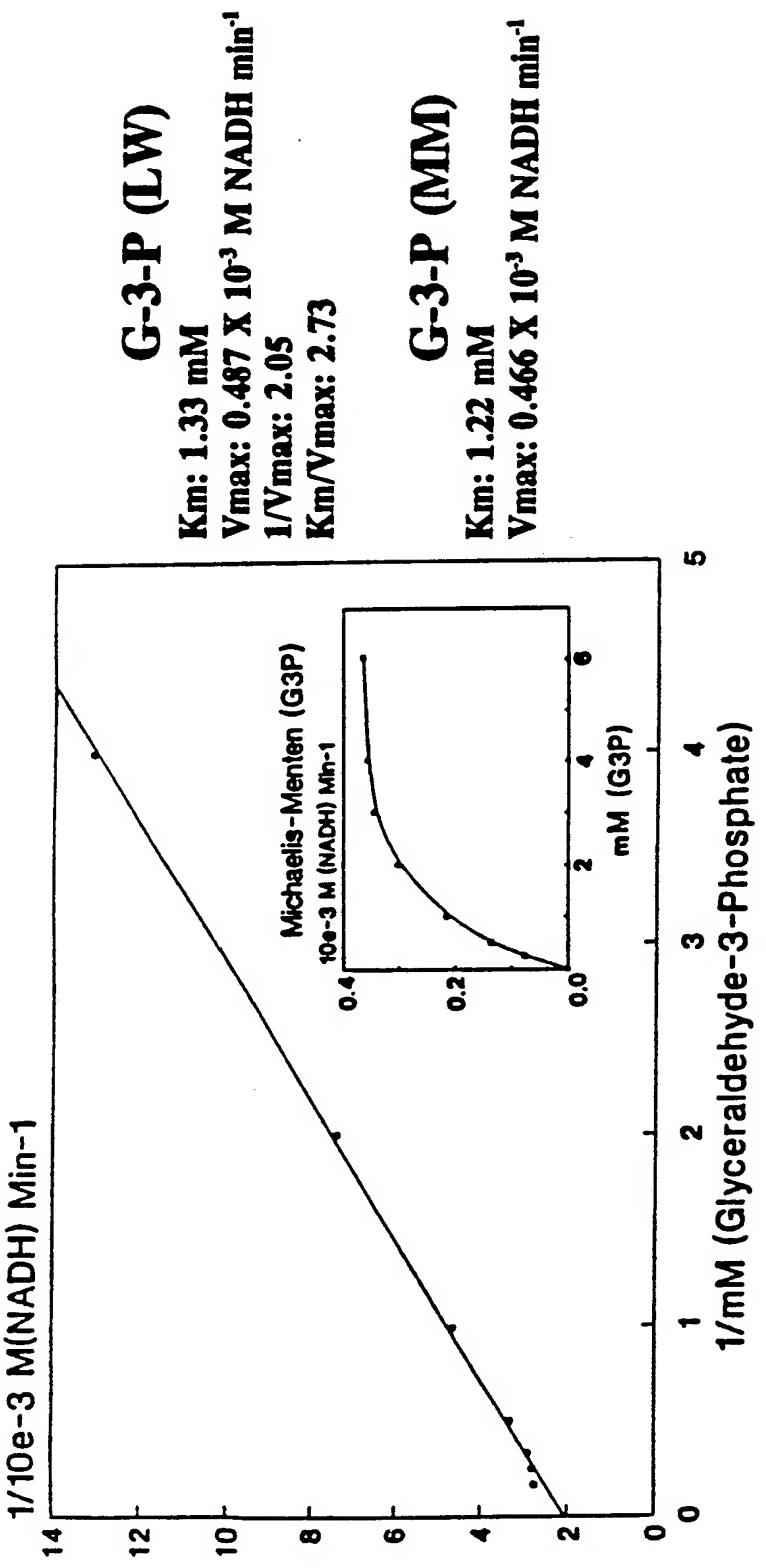
G-3-P (LW)
Km: 1.33 mM
Vmax: 0.487 X 10-3 M NADH min-1
1/Vmax: 2.05
Km/Vmax: 2.73
G-3-P (MM)
Km: 1.22 mM
Vmax: 0.466 X 10-3 M NADH min-1
1/10e-3 M(NADH) Min-1
1/mM (Glyceraldehyde-3-Phosphate)
Michaelis-Menten (G3P)
10e-3 M (NADH) Min-1
mM (G3P)

FIG.3A

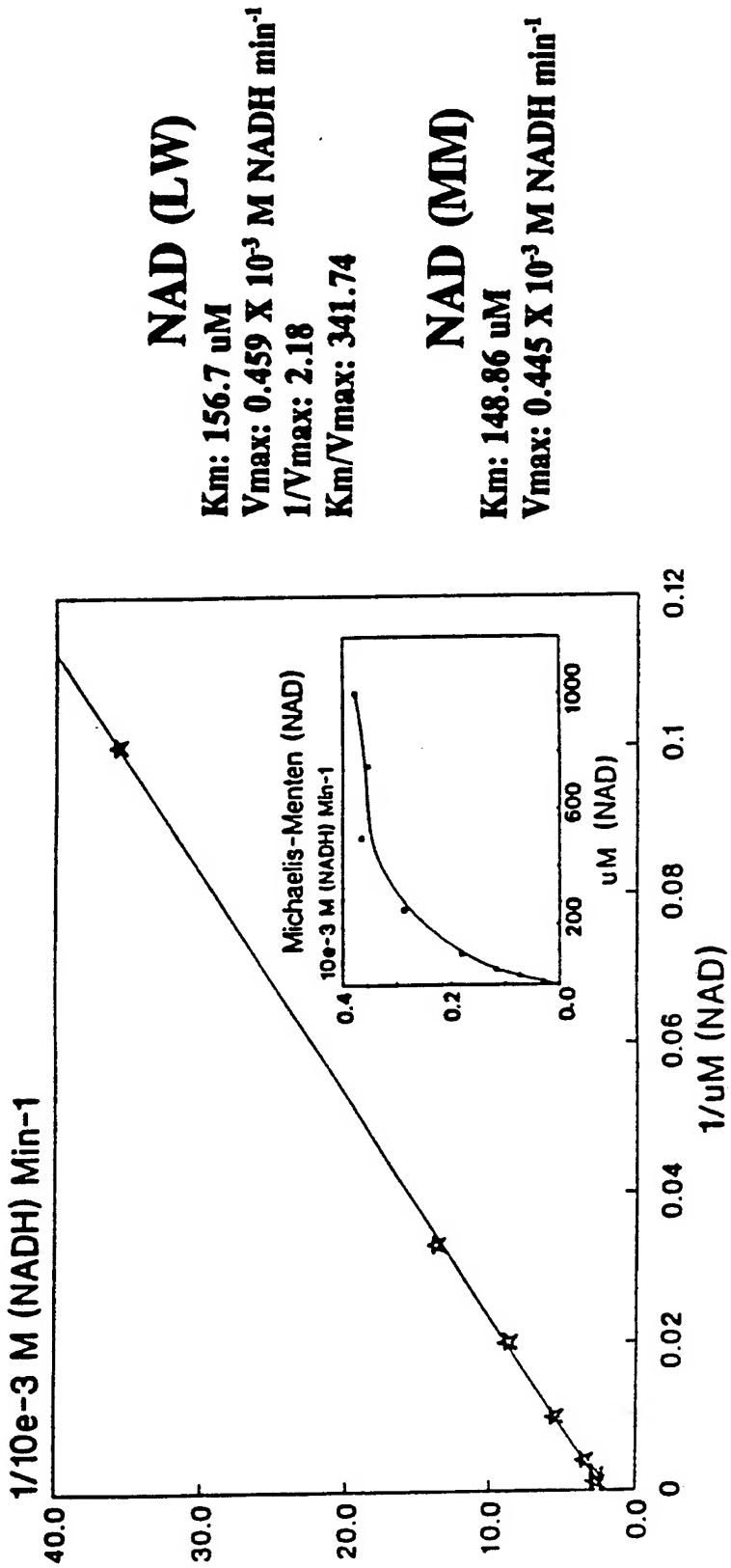
NAD (LW)
Km: 156.7 uM
Vmax: 0.459 X 10-3 M NADH min-1
1/Vmax: 2.18
Km/Vmax: 341.74
NAD (MM)
Km: 148.86 uM
Vmax: 0.445 X 10-3 M NADH min-1
1/10e-3 M (NADH) Min-1
40.0
30.0
20.0
10.0
0.0
0
0.02
0.04
0.06
0.08
0.1
0.12
1/uM (NAD)
Michaelis-Menten (NAD)
10e-3 M (NADH) Min-1
0.4
0.2
0.0
200
600
1000
uM (NAD)

FIG.3B

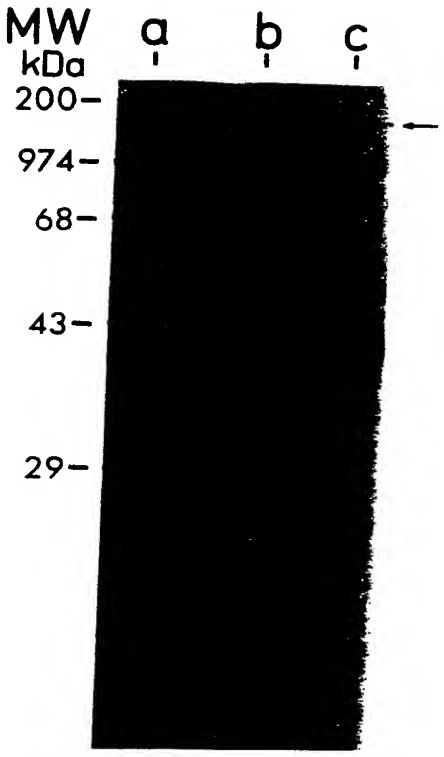

FIG.4A

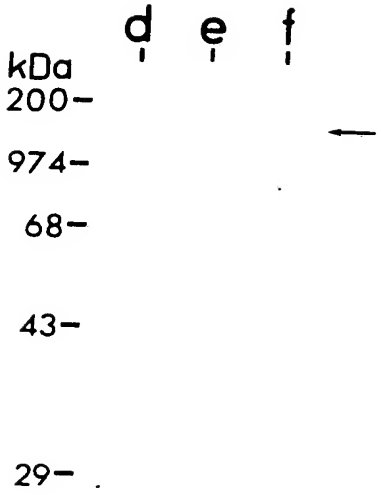

FIG.4B

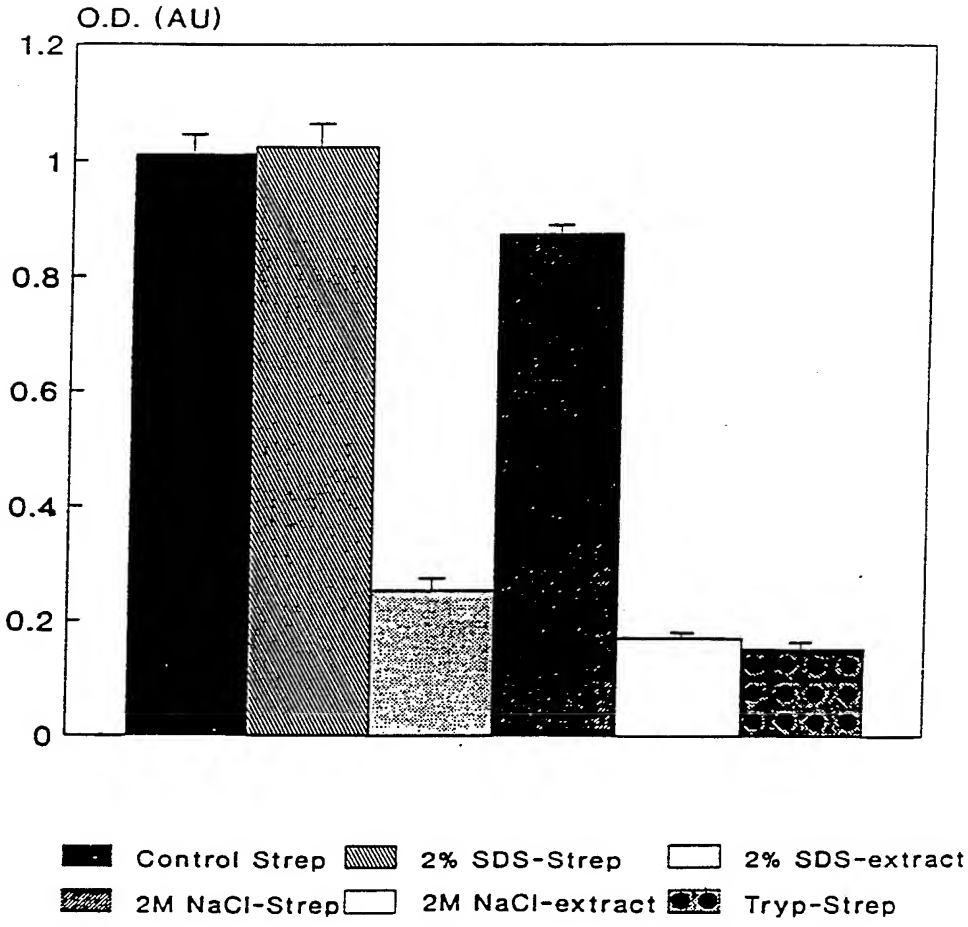
O.D. (AU)
1.2
1
0.8
0.6
0.4
0.2
0
Control Strep
2% SDS-Strep
2% SDS-extract
2M NaCl-Strep
2M NaCl-extract
Tryp-Strep

FIG.5

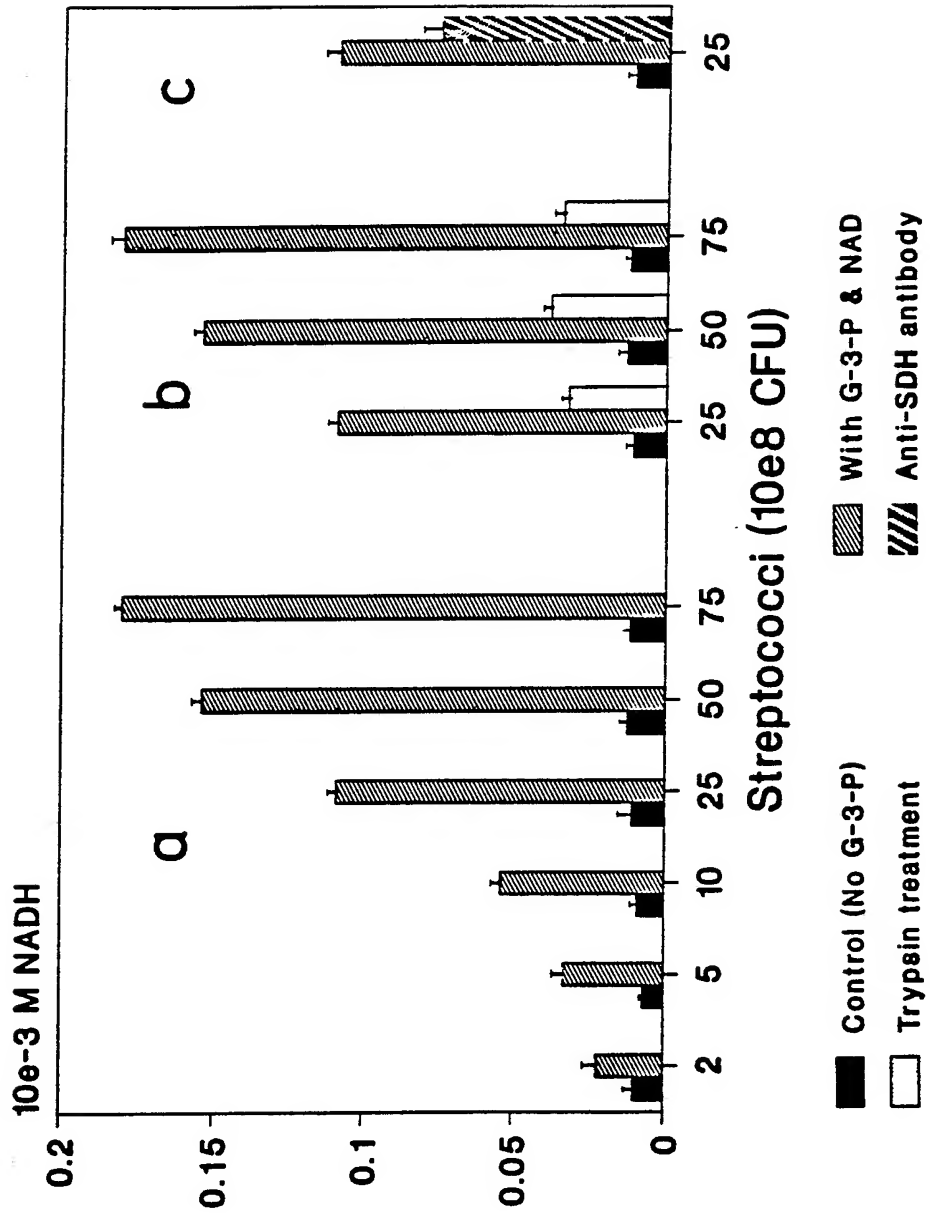
10e-3 M NADH
0.2
0.15
0.1
0.05
0
a
b
c
2
5
10
25
50
75
25
50
75
25
Streptococci (10e8 CFU)
Control (No G-3-P)
Trypsin treatment
With G-3-P & NAD
Anti-SDH antibody

FIG. 6

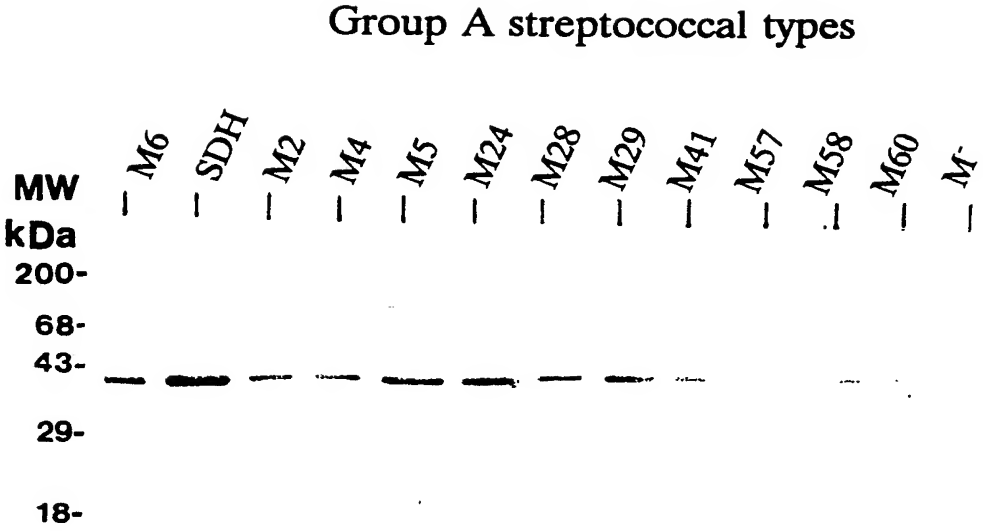
Group A streptococcal types
MW
kDa
200-
68-
43-
29-
18-
M6
SDH
M2
M4
M5
M24
M28
M29
M41
M57
M58
M60
M-

FIG.7A

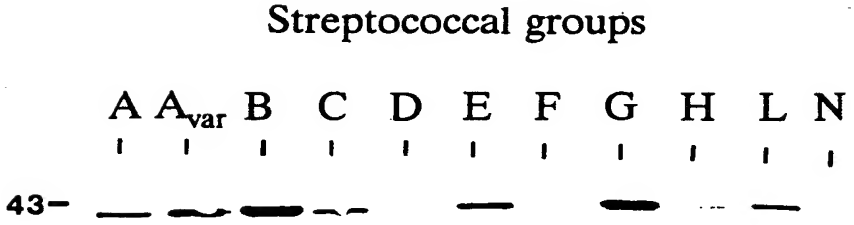
Streptococcal groups
A
A$_{var}$
B
C
D
E
F
G
H
L
N
43-

FIG.7B

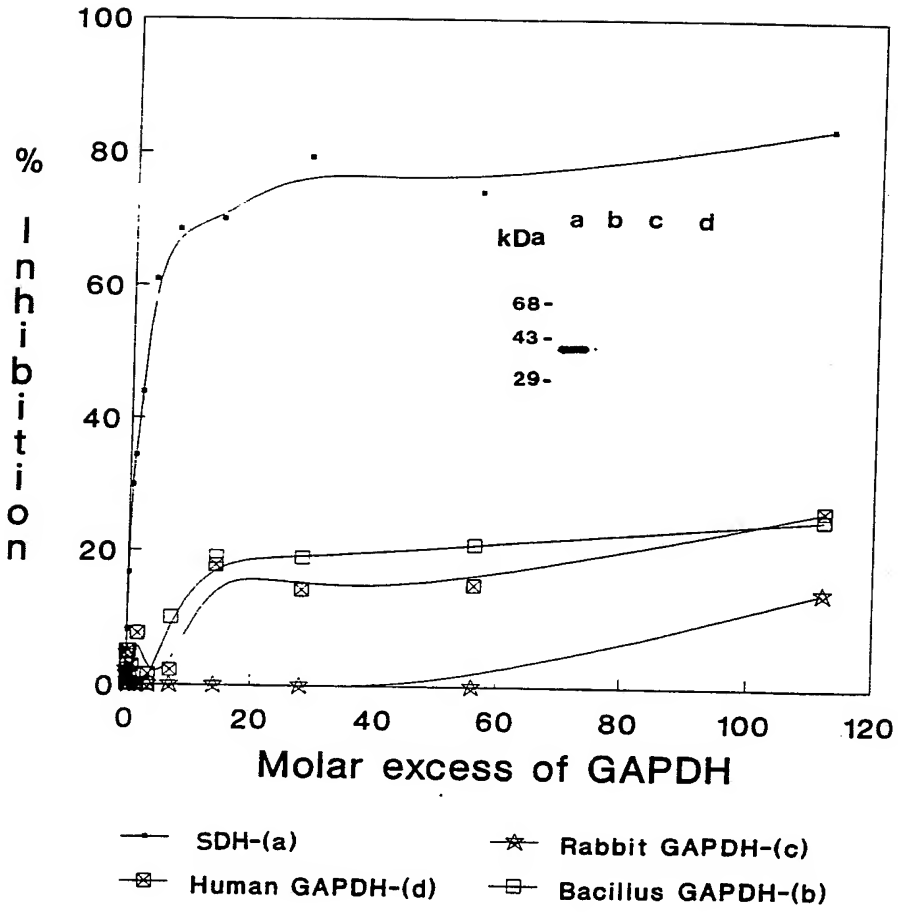
100
80
60
40
20
0
% Inhibition
0
20
40
60
80
100
120
Molar excess of GAPDH
kDa
a b c d
68-
43-
29-
SDH-(a)
Rabbit GAPDH-(c)
Human GAPDH-(d)
Bacillus GAPDH-(b)

FIG.8

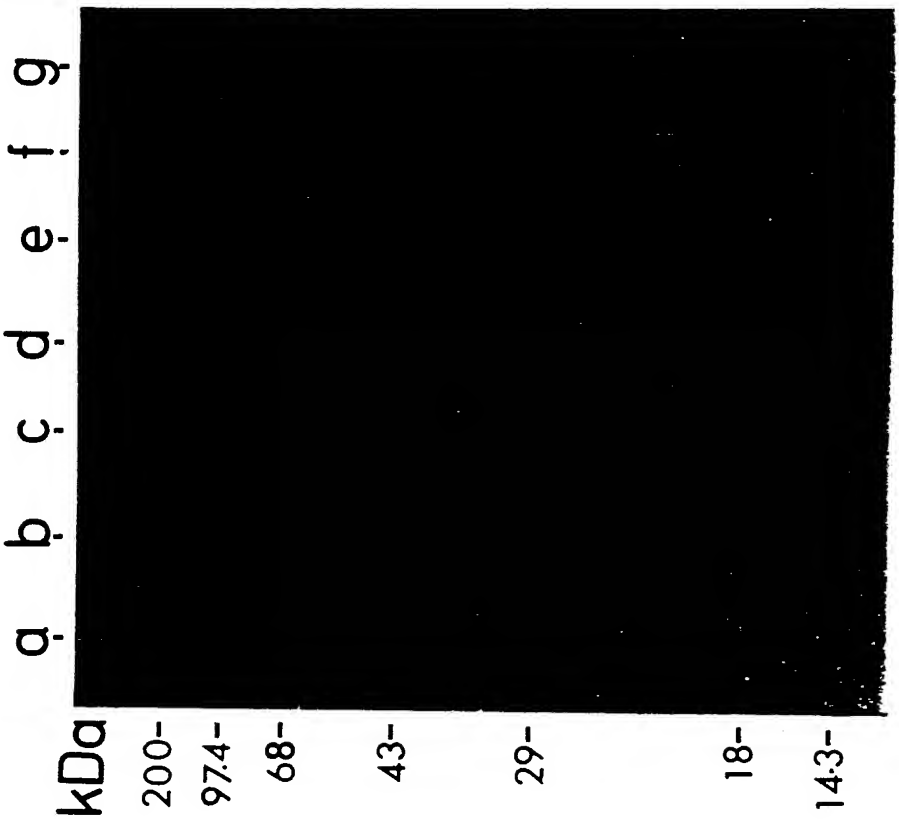
a
b
c
d
e
f
g
kDa
200-
97.4-
68-
43-
29-
18-
14.3-

FIG.9B

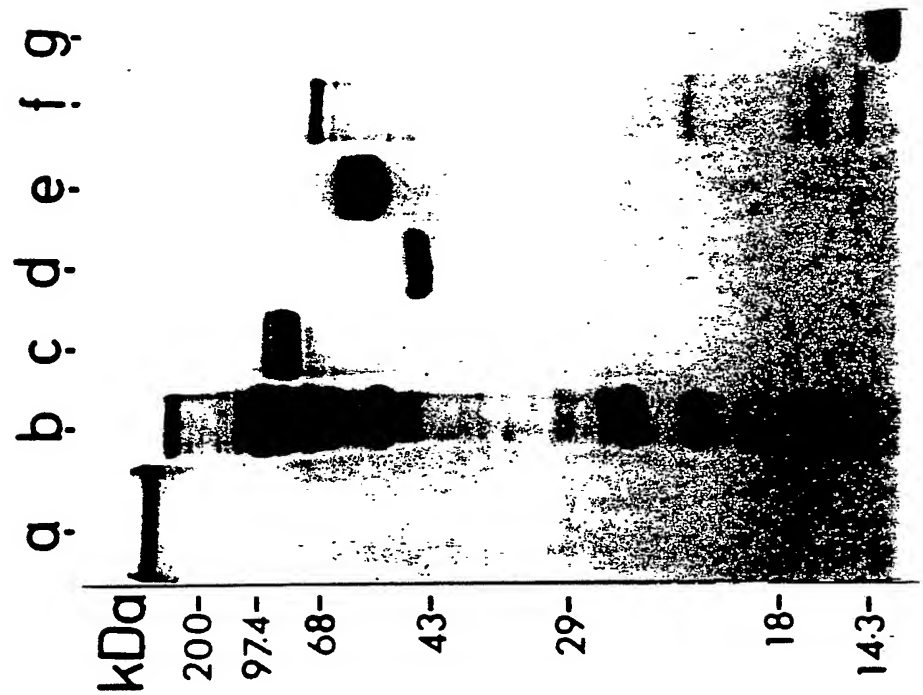
a
b
c
d
e
f
g
kDa
200-
97.4-
68-
43-
29-
18-
14.3-

FIG.9A

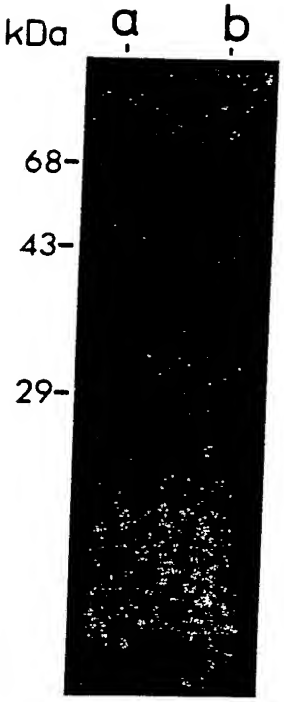

FIG.10A

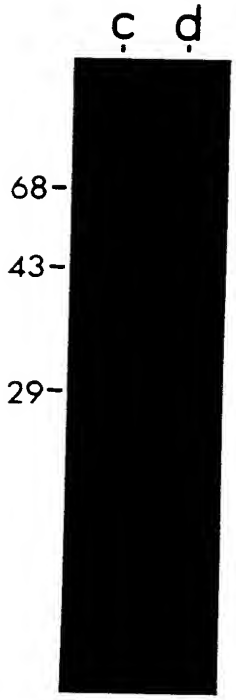

FIG.10B

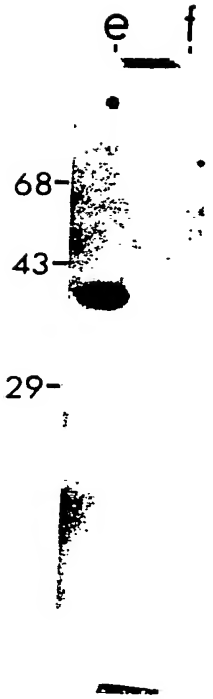

FIG.10C

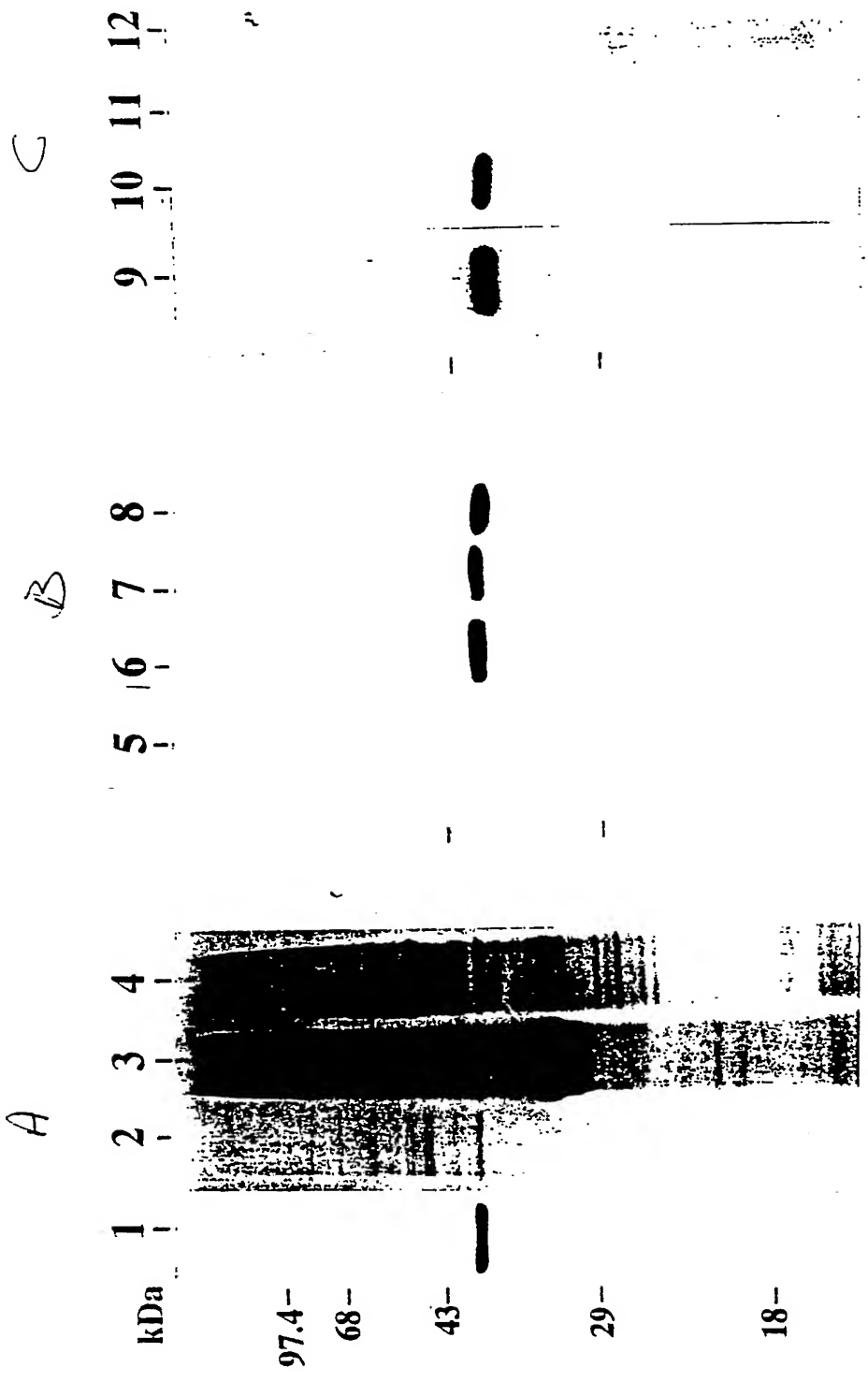
A
B
C
kDa
97.4
68
43
29
18
1 2 3 4
5 6 7 8
9 10 11 12
Coomassie Stain
anti-SDH Ab
Autoradiograph

FIGURE 11

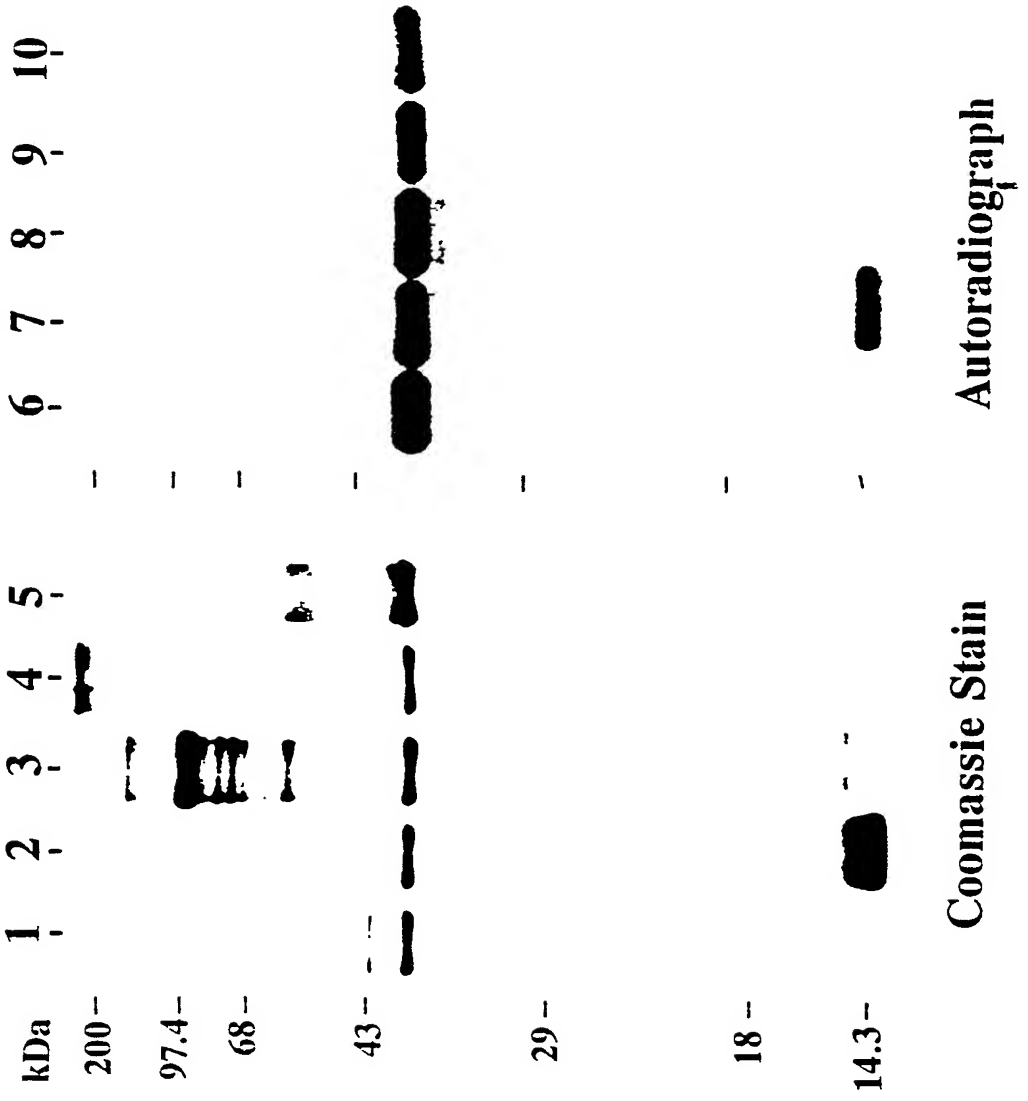
kDa
200
97.4
68
43
29
18
14.3
1 2 3 4 5
6 7 8 9 10
Coomassie Stain
Autoradiograph

FIGURE 12

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/00082

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N9/02; A61K39/09; C12P21/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	ABSTRACTS OF THE ANNUAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY vol. 91, no. B-30, May 1991, WASHINGTON US R.LOTTENBERG ET AL 'A group A Streptococcal plasmin receptor demonstrates homology with Glyceraldehyde-3-phosphate dehydrogenase' see the abstract ---	1-3
A	WO,A,9 015 872 (THE ROCKEFELLER UNIVERSITY) 27 December 1990 see the whole document ---	1-4

-/--

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21 MAY 1993	21 -06- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CUPIDO M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	THE JOURNAL OF EXPERIMENTAL MEDICINE vol. 176, no. 2, 1 August 1992, NEW-YORK US pages 415 - 426 V.PANCHOLI AND V.A.FISCHETTI 'A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate dehydrogenase with multiple binding activity' see the whole document -----	1-4

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9300082
SA 69030

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 21/05/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9015872	27-12-90	AU-A-	5948490	08-01-91
		CA-A-	2035039	22-12-90
		EP-A-	0437604	24-07-91
		JP-T-	3504336	26-09-91